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The goal of our work is to develop novel methods based on rSV5 for killing tumor cells. We have made significant progress in several of the approved tasks. First, we have generated stable tissue culture cell lines which express human HER-2. These cell lines are an important component of our plan to test for specificity of infection through the single chain antibodies (sFv) to HER-2, and will be necessary for titering by plaque assay. Secondly, our previous hybrids composed of the anti-Her-2 sFv linked to fragments of the SV5 HN glycoprotein were found to be very inefficiently transported to the cell surface. These proteins would be poor candidates to replace the normal attachment protein HN, since it is very likely that no infectious virus would be generated. As part of the original task 1, alternative approaches were employed to determine requirements for incorporation of sFv into virions. As such, genes were constructed such that the sFv was attached to the end of full length HN. Alternatively, a gene was made such that the sFv was attached to SH, a second SV5 membrane protein. Immunofluorescence demonstrated efficient cell surface expression of both Hnfull and SH-sFv. Infectious clones harboring these novel attachment proteins have been generated and will be used to generate infectious virus.

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Table of Contents

Introduction

Viruses and virus-based vectors represent a powerful tool for the delivery of recombinant molecules to cells. However, a major drawback to current systems has been the inability to target and limit an infection by a recombinant virus to predetermined cell types or tissue. The goal of the proposed work is to develop a novel method based on recombinant SV5 (rSV5) for targeting and killing predetermined population of tumor cells. The hypothesis to be tested is that the cell-type specificity of an SV5 infection can be pre-determined by incorporating the appropriate foreign membrane protein into the viral envelope. In this work, recombinant SV5 (rSV5) will be engineered to incorporate a membrane-bound form of a single chain antibody (sFv) into its envelope. It is anticipated that the specificity of infection by this rSV5 will be limited to cells expressing the appropriate surface antigen that is recognized by this antibody. To test this hypothesis, virions will be isolated which contain in their envelope an sFv that is specific for HER2, a cell surface antigen that is over expressed in a large number of carcinomas. Cell lines will be created which express HER2. The cell-type specificity of infection by this recombinant virus will be tested to determine if the infection is limited to cancer cells expressing the cell surface HER2 antigen. This annual report describes the problems we have encountered in identifying a candidate attachment protein to insert into the rSV5 genome. We also describe two potential solutions that appear to be promising alternatives to the original proposed approach to targeting rSV5 to preferentially infect HER-2 cells.

Body

List of Approved Tasks

Task 1. To determine the requirements for incorporation of the membrane-bound HER2-specific sFv into the SV5 envelope.

a. Chimeric proteins containing portions of either the SV5 HN or the F protein link the sFv will be analyzed by flow cytometry and by indirect immunofluorescence to identify the construct which provides the most efficient cell surface expression.
b. Incorporation of the chimeric sFv proteins into SV5 virions will be confirmed by expression in SV5-infected cells and western blotting of virions.

Task 2. To determine if the tropism of rSV5-sFv-F is restricted to cells which express cell-surface HER2.

a. Cell lines which contain the HER2 gene under control of an inducible (tetracycline) promoter will be isolated by transfection of plasmids containing a drug resistant gene. Expression in the cell lines will be monitored by immunoblotting with anti-HER2 monoclonal antibodies
b. Recombinant SV5 viruses (rSV5-sFv and rSV5-sFv-stop) that contain the above chimeric genes will be generated. Viruses will be characterized by biochemical and virological approaches.
c. The specificity of infection by rSV5-sFv will be determined by flow cytometic analysis, western blotting of cell lysates and by virus neutralization assays.

Research Accomplished on Task 1.

1. Inefficient cell surface transport of proteins containing the anti-HER2 sFv linked to fragments of the HN protein. The original approach to changing the attachment protein of SV5 involved constructing a series of chimeric proteins which contained a portion of the HN protein linked to the anti-HER2 sFv as diagramed schematically in Fig. 1. The chimeric proteins were efficiently expressed in transfected cells as assayed by western blotting of cell extracts (right panel of Fig. 1).

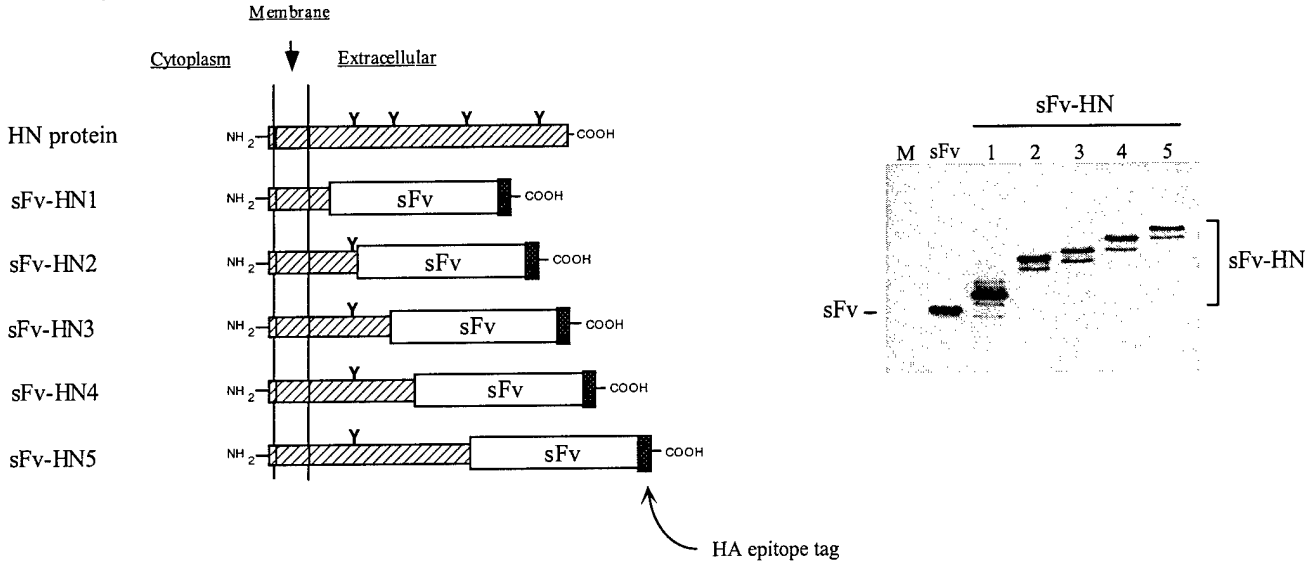

Figure 1. A) Schematic diagram of chimeric proteins containing the anti-HER2 sFv linked to portions of the HN membrane protein. B) Western blot of cells expressing the unmodified sFv or one of the chimeric proteins.

However, as stated in our previous annual report, the level of cell surface expression of the chimeric proteins was very low. This was evident when we attempted to stain transfected cells with the antibody to the HA epitope tag at the c-terminal end of the molecules. This low level of cell surface expression is a problem for the recovery of infectious virus with a new attachment protein, since virus budding occurs at the cell surface and this is where the attachment protein is incorporated into the virion. We have determined that this low cell surface expression is not due to inefficient transfection efficiency, since transfected cells that are permeablized with detergent show very clear staining patterns (not shown). As reported in our previous annual report, our attempts to overcome this problem by removing the naturally occurring HN internalization signal were not successful.

2. Efficient cell surface expression of a chimera linking the anti-HER2 sFv to the full length HN protein. Recently, a recombinant measles virus has been reported that had a new cell tropism by incorporation of a sFv onto the C-terminal end of the H attachment protein (Hammond et al., 2001). Thus, the rationale was that the full length HN protein would be able to fold properly and independently of the sFv, while the truncated HN fragments we had used were malfolded and were not competent for transport to the cell surface.

To determine if a similar approach could be used to overcome the problem with our truncated HN-sFv chimeras, we constructed a cDNA to encode a hybrid protein in which the anti-HER2 sFv was linked to the full length HN. As shown schematically in figure 2, the open-reading-frames for the HN and sFv were separated by a $(Gly\text{-}Gly\text{-}Ser)_3$ spacer region.

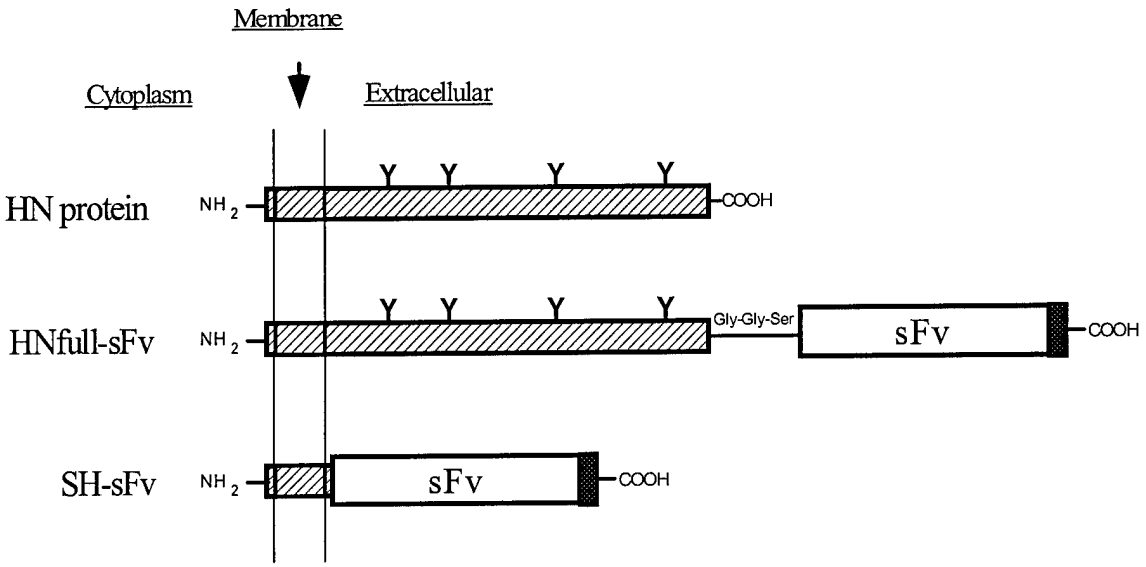

Figure 2. A) Schematic diagram of chimeric proteins containing the anti-HER2 sFv linked to either the full length HN membrane protein or the viral SH protein which has the same orientation in the membrane.

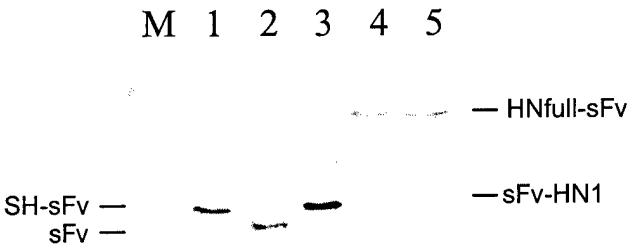

Figure 3. Western blot of extracts from cells transfected with empty vector (M), or with plasmid encoding SH-sFv (lane 1), sFv alone (lane 2), sFv-HN1 truncated (lane 3), or two clones of Hnfull-sFv (lanes 4 and 5). The anti-HA antibody was used to detect proteins.

To moniter expression of the new chimeric protein, cells were transfected with plasmids encoding unmodified sFv, the truncated sFv-HN1 (see fig. 1), or two clones encoding the full length HN linked to sFv. Cell extracts were prepared and analyzed by western blotting with the antibody to the C-terminal HA tag on the sFv. As shown in figure 3, cells transfected with the HNfull-sFv plasmids expressed a high molecular weight protein that was not see in mock transfected cells (lane M). The size of the HNfull-sFv protein was similar to WT HN (not shown).

Immunofluorescence was used to determine if the HNfull-sFv was transported to the cell surface where it could be incorporated into budding virions. CV-1 cells were mock transfected or transfected with plasmids encoding WT HN or the HNfull-sFv chimera. At 20 h post transfection, cells were fixed and analyzed for cell surface staining using a monoclonal antibody specific for the native conformation of HN. As shown in Fig. 4, cells transfected with WT HN plasmid showed bright punctate cell surface staining that was not seen in mock transfected cells. Likewise, cells transfected with the HNfull-sFv plasmids showed bright cell surface staining, however the pattern of staining was not as clear and punctate as that seen in the case of WT HN. The number of cells expressing HNfull-sFv closely matched the WT HN sample. Together, these data are very important for our project, since they demonstrate that the chimeric HNfull-sFv is efficiently expressed at the cell surface where budding can occur.

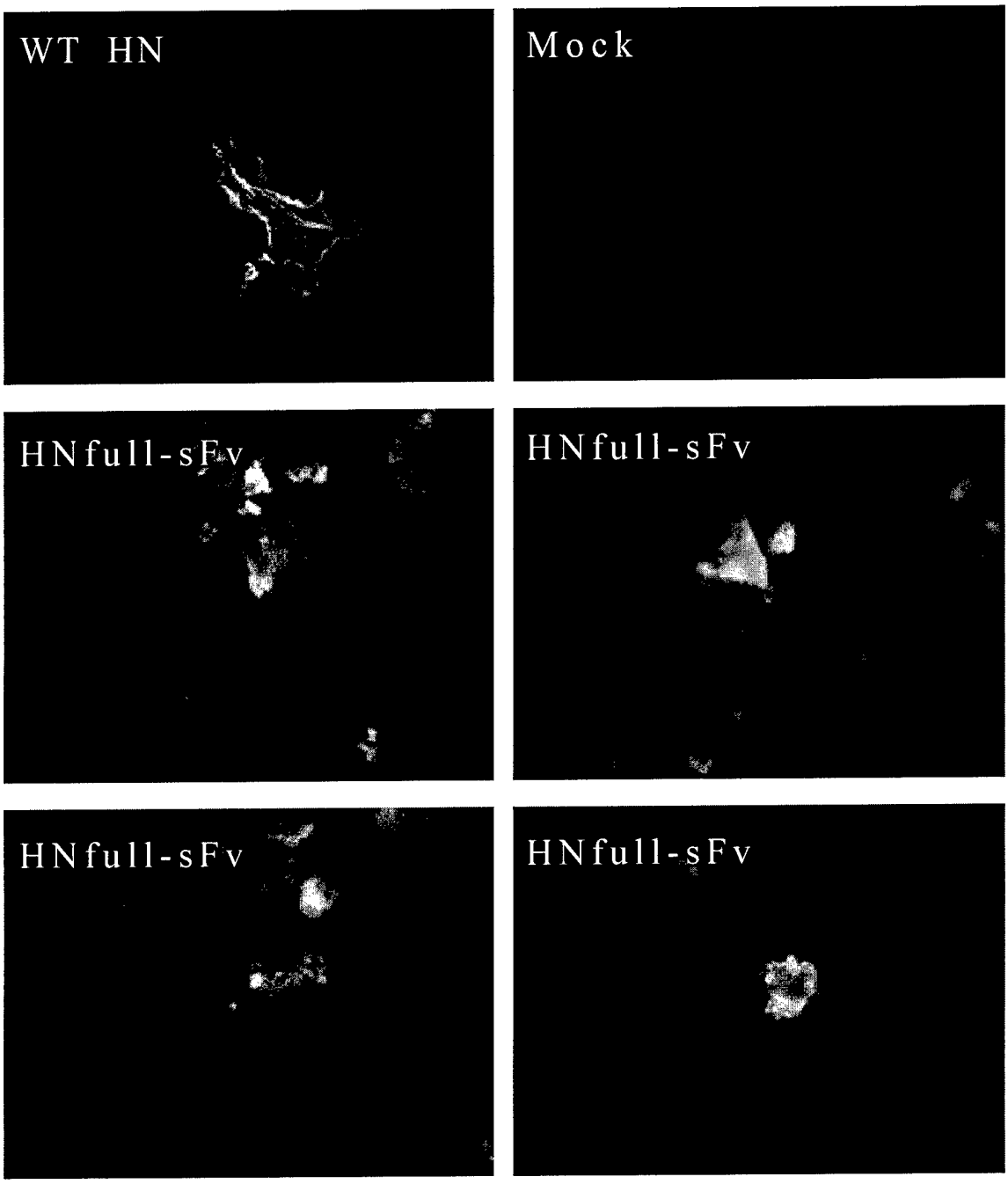

Figure 4. CV-1 cells were mock transfected or transfected with plasmids expressing WT HN or the HNfull-sFv chimeric protein. Cells were fixed in paraformaldehyde and stained with anti-HN antibody followed by FITC-conjugated anti-mouse.

Efficient cell surface expression of a chimera linking the anti-HER2 sFv to the SH protein. As an alternative approach to incorporating the sFv into an SV5 virion, we have constructed a cDNA encoding the sFv linke to the SV5 small hydrophobic (SH) protein. SH is a 44 amino acid protein expressed at the surface of infected cells. The orientation of SH is such that the N-terminal end is in the cytoplasm and only two residues extend into the extracellular space (see Fig. 2 schematic). We reasoned that a membrane protein with such a short ectodomain would have less stringent requirements for transport than that see for the 550 residue HN protein, and thus would be more likely to transport the sFv to the surface. SH is incorporated into budding virions, which would fulfill an additional requirement.

A cDNA clone was constructed to link the sFv open-reading-frame to SH. Western blotting of transfected cells showed efficient expression (see Fig. 3 above). Cell surface staining experiments similar to that shown in Fig. 4 have not yet been confirmed, but preliminary data indicate that transport is very efficient. Future work will analyze the chimeric SH-sFv in parallel with the HNfull-sFv.

Research Accomplished on Task 2.

Isolation of cell lines that constitutively express human HER-2. As described in task 2a, we have set out to isolate cell lines that have tet- inducible expression of HER-2. The previous report described our success in isolating cells that have high expression of the tet repressor needed to block expression of a target gene under control of the tet repressor element. However, despite numerous attempts, we have been unable to isolate double-selected cell lines that express HER-2 under the tet operator. As an alternative approach to create a cell line to propagate and titer the rSV5 containing the HNfull-sFv attachment protein, we have isolated cell lines permissive for SV5 growth and plaque assay that express human HER-2. The HER-2 gene was inserted into pBabe under control of the CMV promoter. DNA was used to transfect CV-1 cells, a cell line that is used for plaquing and growing SV5 stocks. Cell colonies that were resistant to G418 (encoded on the plasmid) were isolated and grow up. As shown in Fig. 5, 4 cells lines were isolate that expressed high levels of HER-2 by western blotting. These are valuable cell lines for our experiments, since they can be used for growth and tittering rSV5. Importantly, along with the parental CV-1 cells, they can also be used to test the specificity of targeted infection by the rSV5-sFv vectors. Thus, Task 2a has been completed.

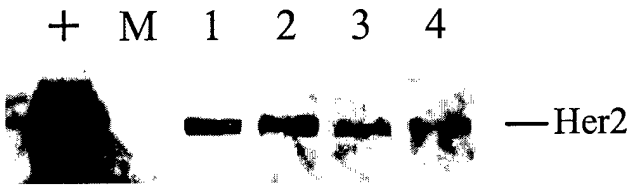

Figure 5. Cell lines constitutively expressing human HER-2. Stable cell lines (lanes 1-4) were isolated by tranfection with a cDNA expressing HER-2 and analyzed by western blotting with a polyclonal anti-HER-2 antibody. M, mock transfected cells; +, HER-2 overexpressed by vaccinia virus infection.

Construction of cDNA encoding HNfull-sFv in place of the WT HN gene. As a first step in recovering virus with an altered attachment protein, we have inserted the gene for HNfull-sFv in place of the WT HN gene. Clones were screened for the presence of a second XbaI site which was introduced as part of the HA tag at the end of the sFv open-reading-frame. As shown in Fig. 6, four clones were identified that produced two XbaI digestion products (asterisks), and the presence of the HNfull-sFv gene was further confirmed by nucleotide sequencing. We are in the process of using the cDNAs to recover infectious virus harboring the modified HN protein. Thus, Task 2b is in progress.

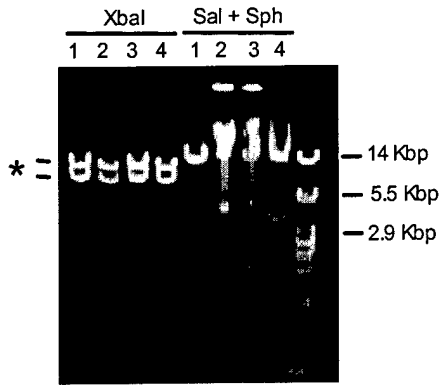

Figure 6. A full length SV5 cDNA with the Hnfull-sFv gene. Colonies were screened for the presence of a new XbaI site which was part of the Hnfull-sFv gene.

Key Research Accomplishments.

- Determined that sFv linked to truncated forms of HN will not be transported to the cell surface at a high enough efficiency to allow incorporation into budding virions.
- Constructed a chimeric protein consisting of the anti-HER2 sFv linked to the full length HN separated by a (Gly-Gly-Ser)3 spacer.
- Determined that the HNfull-sFv protein is very efficiently transported to the cell surface of transfected cells. This will form the basis for the rSV5 with altered attachment protein. **(Task 1a finished)**
- Constructed a chimeric protein consisting of the anti-HER2 sFv linked to the SV5 small hydrophobic protein SH. The SH-sFv protein is expressed at the cell surface as assayed by immunofluorescence. **(Task 1a alternative)**
- Isolated monkey kidney cell lines over-expressing human HER-2 for growing and titering rSV5, and for testing the specifity of infection **(Task 2a finished).**
- Constructed full length infectious cDNA for SV5 that has the HNfull-sFv inserted in place of the normal attachment protein. **(Task 2b in progress)**

Reportable Outcomes

1) Construction of genes encoding membrane-bound, cell surface chimeric protein composed of the anti-HER2 sFv linked to paramyxovirus HN protein separated by Gly-Ser spacer region.
2) Isolation of monkey kidney cell lines constitutively expressing human HER-2.

Conclusions

The overall goal of our work is to develop the paramyxovirus SV5 as a novel vector for controlled and targeted killing of tumor cells. Our model system is based on construction of a rSV5 with a novel attachment protein containing the anti-HER2 sFv linked to HN. The specificity of infection would be tested in cell lines with inducible expression of HER2. We have encountered two problems in our original plan: the proposed sFv-HN fusion proteins with truncated HN were inefficiently transported to the cell surface where budding occurs and we were unable to isolate double-drug resistant stable cell lines with inducible expression of HER2.

In order to move our work forward, we have overcome both of these problems by taking alternative approaches that are still well within the approved Statement of Work. We have constructed a new attachment protein whereby the sFv is linked to the full length HN, not to a fragment of HN. This molecule is efficiently expressed at the surface (see fig. 4 above). In addition, we have linked the sFv to the SH protein, a second SV5 protein that is incorporated into virions and we will use this as yet another alternative approach. Again, this is still within the approved statement of work which was to determine the requirements for incorporating membrane bound anti-HER2 sFv into a virion.

Likewise, we have taken an alternative approach to isolate cell lines expressing HER2. The CV-1 cell lines described in this report are still within the Statement of Work, since they will allow us to test the specificity of infection with the rSV5-HNfull-sFv by using normal CV-1 cells and the HER2 expressing cells as negative and positive target cells.

We have requested a no-cost extension for this project, which has been approved for one year. During the next year we will continue our efforts to recover rSV5 with a new attachment protein. We have in hand a full length SV5 cDNA with the gene for the new attachment protein and are in the process of trying to recover infectious virus through the use of the HER2-expressing cell lines described here. Our work will be an important addition to the development of vectors that are capable of infecting predetermined populations of tumor cells and, together with a suicide gene (e.g., Parks et al, 2002), the rSV5 vectors will be used for controlled killing of tumor cells.

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